Effects of Melatonin on Improvement of Neurological Function in Focal Cerebral Ischemic Rats

Seunghoon Lee[1,#], Jinhee Shin[2], Minkyung Lee[2], Sang-kil Lee[3], Sang-Rae Lee[4], Kyu-Tae Chang[4,†] and Yonggeun Hong[1,2,3,4,†]

[1]*Department Rehabilitation Science in Interdisciplinary PhD Program, Graduate School of Inje University, Gimhae 621-749, Korea*

[2]*Department Physical Therapy, Graduate School of Inje University, Gimhae 621-749, Korea*

[3]*Cardiovascular and Metabolic Disease Center, College of Biomedical Science and Engineering, Gimhae 621-749, Korea*

[4]*National Primate Research Center, Korea Research Institute of Biotechnology, Ochang 363-883, Korea*

ABSTRACT

Acute ischemic stroke results from sudden decrease or loss of blood supply to an area of the brain, resulting in a coinciding loss of neurological function. The antioxidant action of melatonin is an important mechanism among its known effects to protective activity during ischemic/reperfusion injury. The focus of this research, therapeutic efficacy of melatonin on recovery of neurological function following long term treatment in ischemic brain injured rats. Male Sprague-Dawley rats (n=40; 8 weeks old) were divided into the control group, and MCAo groups (Vehicle, MT7 : MCAo+ melatonin injection at 7:00, MT19 : MCAo+melatonin injection at 19:00, and MT7,19 : MCAo+melatonin injection at 7:00 and 19:00). Rat body weight and neurological function were measured every week for 8 weeks. After 8 weeks, the rats were anesthetized with a mixture of zoletil (40 mg/kg) and xylazine (10 mg/kg) and sacrificed for further analysis. Tissues were then collected for RNA isolation from brain tissue. Also, brain tissues were analyzed by histological procedures. We elucidated that melatonin was not toxic in vital organs. MT7,19 was the most rapidly got back to mild symptom on test of neurological parameter. Also, exogenous melatonin induces both the down-regulation of detrimental genes, such as NOSs and the up-regulation of beneficial gene, including BDNF during long term administration after focal cerebral ischemia. Melatonin treatment reduced the loss of primary motor cortex. Therefore, we suggest that melatonin could be act as prophylactic as well as therapeutic agent for neurorehabilitative intervention.

(Key words : Melatonin, Focal cerebral ischemia, Neurological function)

INTRODUCTION

Stroke or cerebrovascular accident (CVA) is the third leading cause of death and a leading cause of long-term disability in worldwide (Feigin, 2005). Brain cells have a relatively high consumption of oxygen and glucose, and depend almost exclusively on oxidative phosphorylation for energy production (Brouns and De Deyn, 2009). Acute ischemic stroke results from sudden decrease or loss of blood supply to an area of the brain, resulting in a coinciding loss of neurological function (Donnan *et al.*, 2008). This immediately leads to dysfunction of energy-dependent ion transport pumps and depolarization of neuronal and glial cells (Martin *et al.*, Katsura *et al.*, 1994). Consequently, voltage-dependent calcium ion channels become activated and excitatory neurotransmitter is released into the extracellular space. Accumulated excitatory neurotransmitter, as glutamate induced the overstimulation of α-amino-3-hydroxyl-5-methyl-4-isoxazole- propionate (AMPA), and N-methyl-D-aspartic acid (NMDA) receptors on the other neural cells, consequent influx of Na^+, Cl^-, and Ca^{2+} through the channels gated by these receptors (Chen *et al.*, 2008). High concentration of Ca^{2+} ion generates a Ca^{2+}-dependent enzyme, including nitric oxide synthase (NOS) and free radicals, especially reactive oxygen species (ROS). Enlarged free radicals lead to acute cell death through necrotic and/or apoptotic cell death (Brouns and Deyn, 2009). Nitric oxide is hydrophilic and hydrophobic free radical. It is generated from L-arginine by three kinds of NOS (Masters, 1994). NOS isoforms have a positive and negative effect in neurodegenerative diseases, including stroke, Alzheimer's disease, and spinal cord injury (Kielstein *et al.*, 2006; Pacher *et al.*, 2007; Brouns and Deyn, 2009). NOS type II (iNOS) well known that it is induced the ca-

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† Corresponding author : Phone: +82-55-320-3681, E-mail: yonghong@inje.ac.kr

spase-mediated apoptosis. Inflammation is a fundamental pathogenic mechanism in stroke. Elevated inflammatory markers predict risk for incident and recurrent stroke. TNF-α also activates NF-κB transcriptional factor, which may increase nitric oxide synthase with subsequent formation of nitric oxide. Nitric oxide plays a major role in ischemic damage evolution and increases in rat brain after focal cerebral ischemia. It not only affects neurons, but also oligodendrocytes and other glial cells. And also, is inducible an apoptosis factor for both sensory and motor neurons. Previous studies demonstrated that IGF-1 treatment inhibits glutamate-, nitric oxide-, and hydrogen peroxide-induced apoptosis (Kooijman *et al.*, 2009), upregulates neurogenesis in the adult hippocampus.

Melatonin, the major secretory product of the pineal gland, has been known to interact with the neuroendocrine axis and circadian rhythms. It has been reported that melatonin acts as a free radical scavenger, antioxidant, and thus as an anti-apoptotic agent (Cuzzocreal *et al.*, 2009). The antioxidant action of melatonin is an important mechanism among its known effects to protective activity during ischemic/reperfusion injury. It is protecting nuclear DNA against cytotoxic by free radicals, membrane lipids and cytosolic proteins from oxidative damage. And also, melatonin reduced the concentration of ROS (Mcacoleod *et al.*, 2005; Cuzzocreal *et al.*, 2009). Finally, melatonin stabilizes cellular membranes and alters activities of enzymes, thereby improving cellular function. Several studies reported that exogenous melatonin has a protective effect against ischemic/reperfusion damage in brain. Also, melatonin suppressed the caspase-mediated apoptosis by oxidative stress. A major previous studies, focus on neuroprotective effect of pre- and short-term treatment melatonin. The focus of this research, therapeutic efficiency of melatonin on recovery of neurological function following long term treatment in ischemic brain injured rats.

MATERIALS AND METHODS

Animals

Forty 8-weeks-old male Sprague-Dawley rats weighing 250 to 300 g were used all studies and obtained from Hyochang Science (Daegu, Korea). The rats were randomly divided the control group, and MCAo groups (Vehicle, MT7 : MCAo+melatonin injection at 7:00, MT19 : MCAo+melatonin injection at 19:00, and MT7,19 : MCAo+melatonin injection at 7:00 and 19:00). They had free access to rodent standard rodent chow (Hyochang Science, Daegu, Korea) and tap water *ad libitum*. All animal procedures were approved by the Ethics Committee for Animal Care and Use in Inje University (Approval No. 2010-21), which is certified by Korean Association of Accreditation of Laboratory Animal Care. All rats were housed two per cage, under controlled environmental conditions (23℃), with an established photoperiod of 12hrs light/dark (light on: 7:00 hr).

Focal Cerebral Ischemia/Reperfusion Surgery

Focal brain ischemia/reperfusion model was induced by the intraluminal suture MCAo method as previously described (Longa and Nagasawa, 1989). All operation was processing under the microscopy (Olympus SZ-61TR, Tokyo, Japan). During the surgical procedure, body temperature was monitored continuously with a rectal probe and maintained at 36.5~37.0℃ with a thermostatically controlled water flow system. Animal were anesthetized with zoletil (40 mg/kg) and xylazine (10 mg/kg) i.p.. The right CCA, ICA, and ECA were exposed through a midline incision of the neck. The ECA was dissected further distally after ligated and cauterized along with the terminal lingual and maxillary artery branches, which were then divided. A 5 cm length of 4-0 monofilament nylon suture, its tip rounded by heating near a flame, and coated the silicone, was used as an occluder and was inserted via the ECA (ECA route). The occluder was advanced into the ICA from 18 to 20 mm beyond the carotid bifurcation. Mild resistance indicated that the occluder was properly lodged in the anterior cerebral artery and thus blocked blood flow to the middle cerebral artery (MCA). The 6-0 silk suture around the ECA stump was tightened around the intraluminal nylon suture to prevent bleeding. The procedure was finished the suture of muscle, skin. Reperfusion, 4-0 nylon was pulled the back off a 1 cm after 1 hr.

Melatonin Treatment

After being focal cerebral ischemia by MCAo surgery, rat were injected melatonin (Sigma-Aldrich, St. Louis, USA) which was dissolved in ethanol (10 mg/kg of body weight/day) (Pei *et al.*, 2004; Yasuo *et al.*, 2007) via subcutaneous at 7:00 (MT7), 19:00 (MT19), 7:00 and 19:00 (MT7,19).

Measurement of Neurological Dysfunction

In all animals, a battery of behavioral tests was performed before MCAo and at 1day, 1, 2, 3, 4, 5, 6, 7, and 8 weeks after MCAo by an investigator who was blinded to the experimental groups. The battery of tests consisted of the modified neurological severity score (mNSS) (Chen *et al.*, 2001). Table 1 shows a set of the mNSS. Neurological function was graded on a scale of 0 to 18 (normal score: 0, maximal deficit score: 18). The mNSS is a composite of motor, sensory, reflex, and balance tests. In the severity scores of injury, 1 score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe is the injury.

2% 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

The TTC (Sigma-Aldrich, St. Louis, MO, USA) solution (2% by weight) was prepared with 37℃ phosphate buffer (0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4, pH 7.4~7.6) immediately before use. The brain was removed, and immediately 2-mm-thick sections were cut 2, 4, 6, 8, 10 mm from the frontal pole with a rodent brain matrix (RBM-4000C, ASI

Table 1. Detailed description of the items forming the modified neurological severity score (mNSS)

Motor Tests	6
Rasing rat by tail	3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° to vertical axis within 30 sec.	1
Placing rat on the floor (normal=0, maximal=3)	3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Fall down to paretic side	3
Sensory Tests	2
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal=0, maximum=6)	6
Balance with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, spins on beam (>60 sec.)	3
Attempts to balance on beam but falls off (>40 sec.)	4
Attempts to balance on beam but falls off (>20 sec.)	5
Attempts to balance on beam but falls off (<20 sec.)	6
Reflex absence and abnormal movements	4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizure, myoclonus, myodystony	1
Maximal scores	18

1~6 scores: mild injury, 7~12 scores : moderate injury, 13~18 scores : severe injury (modified from reference 17)

instruments, Warren, MI, USA). The brain tissues were put in 2% TTC solution contained dishes. The dishes were covered with aluminum foil to prevent exposure to light and incubated at 37℃ for 30 minutes. The TTC solution was then replaced with 10% buffered formalin. To prevent distortion, brain slices were kept flat in the Petri dish overnight. The stained sections were photographed within 3~7 days (Bederson *et al.*, 1986; Isayama *et al.*, 1991).

H and E Staining

The brain was fixed by intracardiac perfusion of 4% NBP (neutral buffer paraformaldehyde) (pH 7.4) after blood removed the using of 1× PBS (phosphate buffer saline). The tissues were embedded using with Tissue-Tek® OCT compound (Miles Laboratory, Elkhart Ind., USA). Rapidly submerge the mold into isopentane cooled with liquid nitrogen. After trimming, tissues was carefully cutting (section thickness: 10 μm), use a small camel hair brush to guide the section off the block face and transfer it to a gelatin subbed slide. Allow the section to dry on the slide at room temperature for 15 minutes (Urushiyama *et al.*, 2004).

Gene Analysis

The brain tissues were homogenized with 1ml of Tri-rea-

gent (Sigma-Aldrich, St. Louis, MO, USA) to prepare a total RNA samples. The RNA was reverse transcribed with oligo d (T) 12~18 using M-MuLV RT (Gibco, BRL) and this reaction mixture served as a template for polymerase chain reaction (PCR). To identify iNOS and nNOS transcription, a reaction mixture (50 μl) for PCR was made up of 2.0 ul of cDNA synthesis mixture, 40 nM dNTPs, 10 pM of sense and antisense primer, and 1.25 U of Taq polymerase (Promega Corporation, Madison, WI, USA). PCR were performed with denaturation at 95℃ for 30 sec, annealing at 60 ℃ for 1 min, and extension at 72℃ for 1min in each cycle, followed by a final 10 min extension at 72℃ using a Takara Thermal Cycler MP, TP 3000.

Statistical Analysis

Data were collected from repeated experiments and are presented as means±SD. A one-way ANOVA was used for statistical analysis. Differences were deemed to be statistically significant when $p<0.05$. All analyses were performed using the SPSS software (SPSS ver. 18.0, IBM, Chicago, IL, USA).

RESULTS

Physiological General Feature of MCAo rats

Body Weight after MCAo

We compared with the value of except initial body weight from final body. MT7,19 group was recovered to similarly body weight with control group. Likewise, MT7,19 was significantly enhanced the body weight more than both MT7 ($p<0.05$) and MT19 ($p<0.01$). Variation of time course, melatonin treatment groups were significantly inhibited the increasing of body weight rather than control group ($p<0.01$) (Fig. 1).

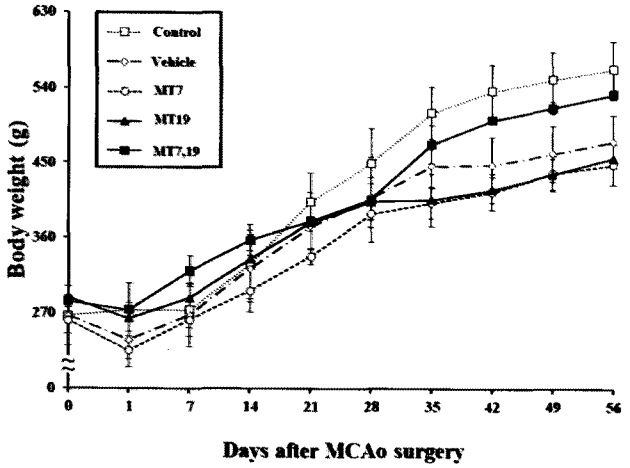

Fig. 1. Change of body weight after intervention of each other condition for 8 week. Melatonin was injected on times, per daily after MCAo surgery during 8 week. MCAo+vehicle, Vehicle; MCAo+melatonin inj. at 7:00, MT7; MCAo+melatonin injection at 19:00, MT19; MCAo+melatonin injection at 7:00 and 19:00, MT7,19. Data were shown as the mean±SD. MCAo=middle cerebral artery occlusion.

Serum Components after MCAo

In the toxicity test of melatonin, the rats did not show any signs of toxicity or changes in general behavior or other physiological and biochemical parameters. No significant changes in the weight of internal organs were observed in melatonin treated rats which implicates that melatonin was not toxic in these vital organs (data not shown).

Alteration of Neurological Dysfunction

Behavioral test was 24 hr after MCAo surgery, once an every week for 8 week using mNSS test. Melatonin treated groups were recovered a neurological symptom faster than vehicle. Especially, MT7,19 group was recovered to mild injury score at 14th day after melatonin treatment. MT7 and MT19 groups were shown a similarly pattern with MT7,19. Conversely, vehicle rat was processing the neurological dysfunction from 1st to 14th day after focal cerebral ischemia. Comparison of melatonin treated groups, MT7,19 was the most rapidly got back to mild symptom (Fig. 2). These results suggest that melatonin might be repressed the processing of apoptotic cell death cascade after focal cerebral ischemia, Thereby melatonin treated rats were recovered to neurological function.

Measurement of Infarct Volume

After 8 week, we measured the infarct volume using 2% TTC staining in ischemic damage lesion. The brain slices were quantified using Image-J analysis software for comparison of cerebral infarct volume (Fig. 3A). The brain of

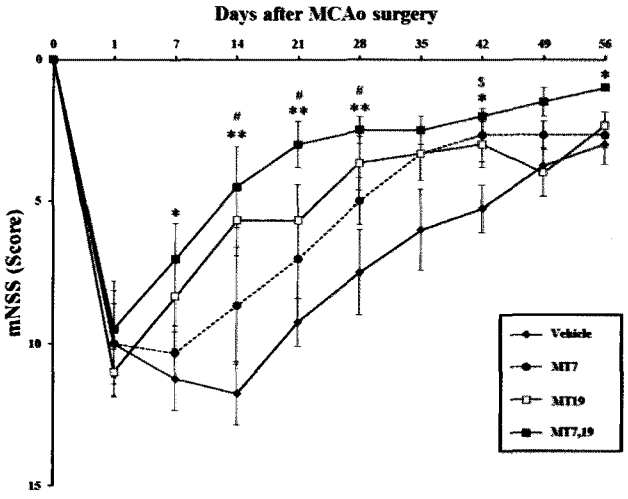

Fig. 2. The assessment of neurological deficiency in MCAo rats. mNSS were measured at 24 hr, per week after MCAo surgery. Data were shown as the mean±SD. * $p<0.05$ and ** $p<0.01$: vs. vehicle, $^{\#}$ $p<0.05$: vs. MT7, $^{\$}$ $p<0.05$: vs. MT19.

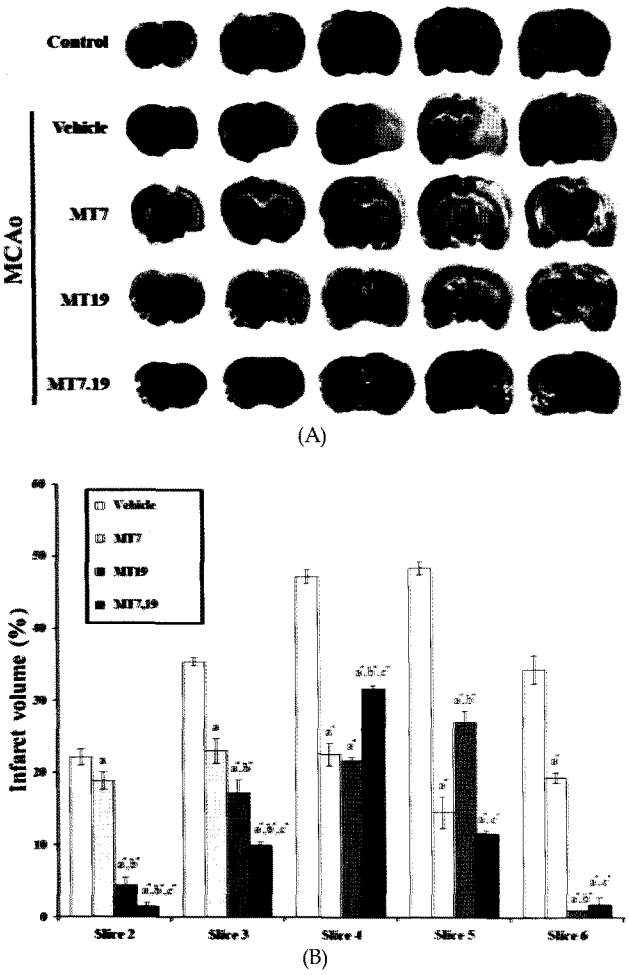

Fig. 3. 2% TTC-defined volume of ischemic lesion after MCAo surgery. (A), TTC-stained images of rats brain slices. Brain of control was intact whole brain section, however, MCAo rats brains were shown a white color (infarction) in right area. (B) Quantification of infarct volume using image-J software. [a] $p<0.05$, [a*] $p<0.01$: vs. vehicle; [b] $p<0.05$, [b*] $p<0.01$: vs. MT7; [c] $p<0.05$, [c*] $p<0.01$: vs. MT7,19.

vehicle rat showed the widest lesion from bregma −0.12 to −2.92 districts. All of melatonin treated groups were significantly decreased an infarct volume more than vehicle ([a]$p<0.05$, [a*]$p<0.01$). In 4[th] slice, MT7,19 was shown the widest region in melatonin treatment groups. There might be showed a vestige of infarct lesion after focal cerebral ischemia (Fig. 3B). Interestingly, infarct volume was showed an important result by melatonin treated times. As a result, there might be maintained higher concentration of melatonin in plasma during 24 hr.

Alteration of Brain Morphology

Histological examination of brain morphology confirmed in bregma −2.92 mm district of rat brain atlas. The focal cerebral ischemic rats showed a loss of neuronal cell counter in CA1 area (data not shown). However, long term melatonin treated rats inhibited a loss of neuronal cell counter.

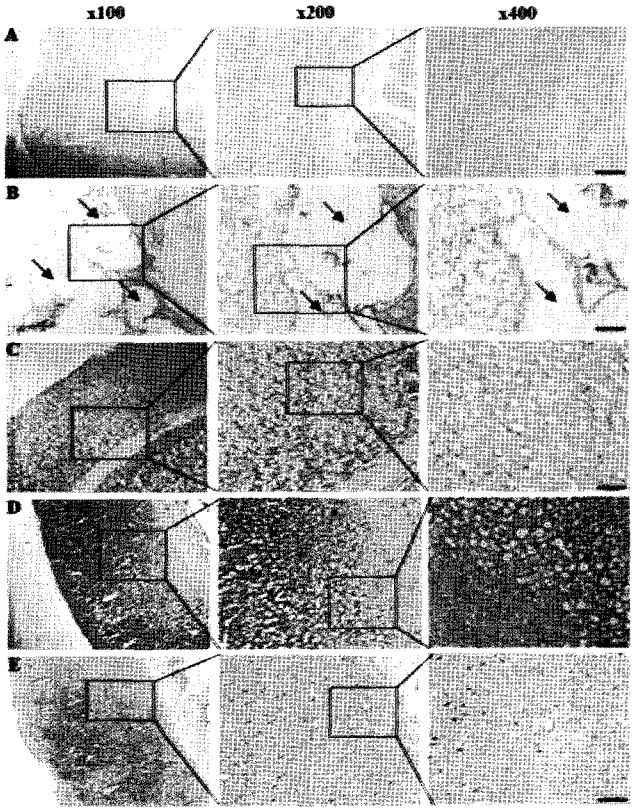

Fig. 4. H and E stained sections of the brain. bregma −0.12 mm area of rat brain atlas. (A), primary motor cortex of control; (B), primary motor cortex of vehicle; (C), primary motor cortex of MT7; (D), primary motor cortex of MT19; (E), primary motor cortex of MT7,19. Scale bars=10 μm. MCAo=Middle cerebral artery occlusion.

In addition, vehicle was lost a primary motor cortex (Fig. 4). Whereas, melatonin treated groups reduced a loss of neuronal cells lesser than vehicle in primary motor cortex. However, intracapsular area not changed (data not shown). Thus, we suggest that long term melatonin treatment might be affected to neuroprotective effect against ischemic/reperfusion-induced dell.

Expression of Detrimental and Beneficial Genes in MCAo brain

NO was generated by NOSs. NO well-known the detrimental effect on ischemic brain injury, Therefore, we analyzed the expression of NOSs mRNA. In vehicle and MT19 rat brains, both iNOS and nNOS were showed the overexpression of mRNA level more than control rat ($p<0.01$). Whereas, MT7 and MT7,19 were significantly reduced the iNOS and nNOS mRNA levels more than vehicle ($p<0.01$). These results suggest that exogenous melatonin might be induced the downregulation of nitric oxide synthesis by calcium-dependent enzymes, such as iNOS, and nNOS. Thereby, melatonin could be closed off the process of secondary damage (Fig. 5A). Next, we analyzed the neurotrophic factor, such as BDNF. Vehicle was significantly revealed the expression

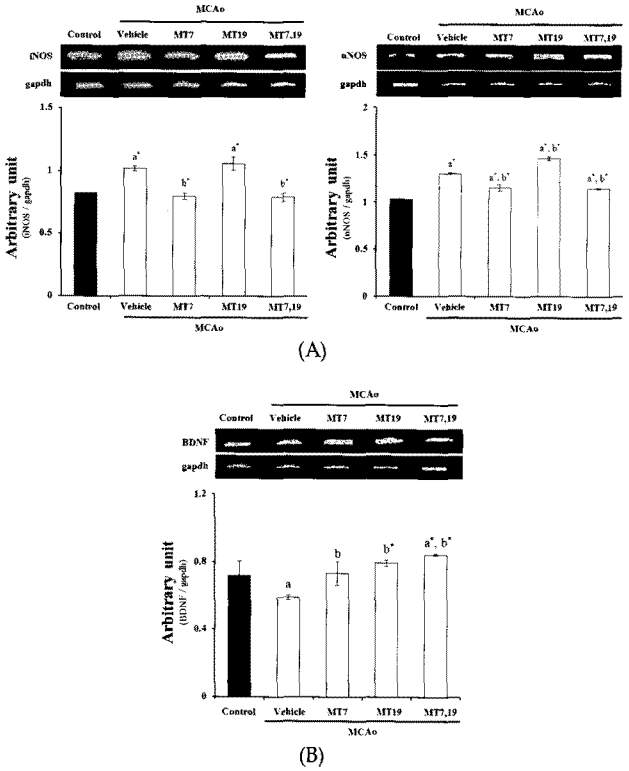

Fig. 5. Detrimental and beneficial effects of melatonin in MCAo rat brain after 8 week. (A) iNOS and nNOS mRNA expression. (B) BDNF mRNA expression. Contol; MCAo+vehicle, vehicle; MCAo+melatonin injection at 7:00, MT7; MCAo+melatonin injection at 19:00, MT19; MCAo +melatonin injection at 7:00 and 19:00, MT7,19. ^{a*} $p<0.01$: vs. control, ^{b*} $p<0.01$: vs. vehicle.

of BDNF lesser than control ($p<0.05$). All melatonin treated groups were dramatically increased more than vehicle (MT7: $p<0.05$) (MT19, MT7,19: $p<0.01$) (Fig. 5B). These results mean that long-term melatonin treatment could be leaded to recovery of neurological function by the increment of neurotrophic factor after focal cerebral ischemia.

DISCUSSION

Recent studies reported that exogenous melatonin have a protective effect of neuronal recovery after central nerve system damage. This study investigated how melatonin affects to an event of morphological changing during focal cerebral ischemia. According to a previous study, pre-treatment of melatonin in focal cerebral ischemia prevented an apoptotic cell death (Kernan and Inzucchi, 2004), because melatonin inhibits the apoptotic signal through the activation of Raf-MEK-ERK (Dai *et al.*, 1998; Erkanl *et al.*, 2004; Koh, 2008). TGF-β reported to be activated in focal cerebral ischemia (Erkanl *et al.*, 2004), suggesting stimulation of apoptosis by inflammatory reaction in focal cerebral ischemic rats. Likewise, we confirmed that melatonin administration inhibits the expression of TGF-β mRNA level after focal cerebral ischemia (data not shown). It means that melatonin treatment might be affected to restrain of augmented apoptosis signaling pathway on ischemic brain after focal cerebral ischemia. Histological data showed that vehicle rats were loosed the primary motor cortex on infarct lesion after 8 weeks. However, melatonin treated groups shown a residual region more than vehicle after 8 weeks. In CA1 region, ipsilateral lesion of vehicle rat was reduced a count of neuronal cell rather than brain of control rats (data not shown). Conversely, melatonin treated rats were reduced a count of died neuronal cell. We found that long-term melatonin treatment retarded the progressing of neural cell death in primary motor cortex and CA1 area of ischemia lesion. Therefore, we suggest that long-term melatonin treatment might be affected to rescue and prevention of neuronal cell death in focal cerebral ischemic rats.

Nitric oxide (NO), a free radical with signaling functions in the CNS, is implicated in some developmental processes, including neuronal survival, precursor proliferation, and differentiation. However, neuronal and inducible nitric oxide synthase (nNOS and iNOS)-derived NO play to opposite role in regulating neurogenesis in the dentate gyrus after focal cerebral ischemia (Corsani *et al.*, 2008; Sehara *et al.*, 2006). Thus, we analyzed the expression of NOSs mRNA on from bregma −0.12 to −2.92 mm area of ipsilateral lesion in brain. The level of iNOS mRNA was enhanced in both vehicle and MT7 rat brain. Conversely, MT7,19 was decreased the level of iNOS mRNA. Likewise, the lecvel of nNOS mRNA was increased in both vehicle and MT19 rat brain. Thereby, we suggest that exogenous melatonin might be revealed the beneficial effect following concentration of plasma.

Previous studies reported that BDNF is a critical factor for brain development, affected to cell survival on brain by melatonin administration after hypoxic damage *in vivo* and *in vitro* (Koriyamna *et al.*, 2009). We found that BDNF mRNA level was decreased on lesion of vehicle rat brain ($p<0.01$). Exogenous melatonin induced the upregulation of BDNF mRNA level in ischemic lesion after 8 weeks. Thereby, we suggest that long term melatonin treatment might be affected to activation of retrieval machinery for restoration of neurological function on ipsilateral lesion after focal cerebral ischemia.

Accordingly, we found that melatonin has two kinds of importantly effects. First, melatonin acts to prevention against NO-mediated apoptotic cell death. Second, melatonin can be turn on the machinery for restoration of neurological function after focal cerebral ischemia. As a result, we expect that melatonin might be used a candidate agent for neuro-rehabilitative approach in stroke.

Several studies reported that administration of melatonin reduced a body weight in mid-age rats (Wolden-Hanson *et al.*, 2000; Speak and Parkin, 1985). Previous study we demonstrated that melatonin was not affect to food consumption on controlled photoperiod in juvenile rat development (Lee *et al.*, 2010). However, present study confirmed that

melatonin administrated-focal cerebral ischemic rats showed growth retardation in body weight. However, MT7,19 was similarly increased a body weight with control rat. Wonden-Hanson *et al* (2000) suggested that body weight could be related to the increased core body temperature induce by melatonin. Lipoprotein lipase (an enzyme required for the uptake of fatty acids into adipocytes) has been shown to have increased activity when incubated at lower temperature. Therefore, melatonin administration keeps on normal core temperature. Thereby, accumulation of adipose tissue might be decreased in visceral organs. Our data show that almost similarity result with previous study. Thus, we suggest that melatonin administration could be acted to the increasing of core temperature. In future study, we will study to relationship between concentration of melatonin in plasma and contents of visceral adipose tissue.

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